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Antibacterial cathelicidin peptide CAP11 suppresses the anandamide production from lipopolysaccharide-stimulated mononuclear phagocytes

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Abstract The action of antibacterial cathelicidin peptide CAP11 on the anandamide production from mononuclear phagocytes was examined. Lipopolysaccharide (LPS)-stimulation induced the anandamide production from macrophage-like RAW264.7, accompanied with the enhanced anandamide-synthesizing enzyme activity; however, the anandamide-degrading enzyme activity was not changed by LPS-stimulation. Importantly, CAP11 suppressed the LPS-induced anandamide production and the increase of anandamide-synthesizing enzyme activity. Furthermore, CAP11 abrogated the LPS-binding to CD14-positive RAW264.7. These observations indicate that CAP11 inhibits the binding of LPS to CD14-positive mononuclear phagocytes, thereby suppressing the anandamide synthesizing enzyme activity and the anandamide production from the cells.

Keywords: Cathelicidin; Antibacterial peptide; Anandamide; Mononuclear phagocyte; Lipopolysaccharide

1. Introduction

Lipopolysaccharide (LPS), also called endotoxin is a major component of the outer membrane of Gram-negative bacteria, and is the most potent activator of mononuclear phagocytes (macrophage and monocyte). LPS triggers mononuclear phagocytes to secrete TNF-α, IL-1β, IL-6 and other mediators such as endogenous cannabinoids, a new class of lipid mediators [1,2]. Anandamide is one of the potent agonists for cannabinoid receptor [3,4]. Anandamide induces the characteristic blood pressure response that leads to hypotension and often bradycardia, via a cannabinoid CB1 receptor and a vanilloid TRPV1 (transient receptor potential vanilloid 1) receptor [5,6]. Thus, anandamide is regarded as a key component that plays a role in the pathoformic stage of Gram-negative bacterial endotoxin shock [2].

To date, several strategies including neutralizing antibodies of LPS, cytokines, and other related signal molecules have been tested to regulate the signal cascade of endotoxin shock [7,8]. Among these, much attention has focused on the low-molecule-weight cationic antibacterial polypeptides that possess both the antibacterial and LPS-neutralizing activities [9–11]. The peptide-based defense against microbial invasion is comprised of two distinct groups of antimicrobial peptides, cathelicidins and defensins, which have been identified in several epithelial tissues and in the granules of phagocyte [12,13]. Previously, we isolated CAP11 (cationic antibacterial polypeptide of 11-kDa), an α-helical cathelicidin peptide, from guinea pig neutrophils, and revealed that CAP11 has the potent abilities to not only kill Gram-positive and -negative bacteria but also neutralize LPS; CAP11 can inhibit the binding of LPS to CD14-positive cells and protect mice from endotoxin shock [11,14–16]. Thus, it could be hypothesized that CAP11 may regulate the production of anandamide, a key inducer for endotoxin shock, from mononuclear phagocytes by affecting the LPS-binding to the cells. In this study, to evaluate the effect of CAP11 on anandamide production, we measured the anandamide production, and the activities for anandamide-producing enzyme and -degrading enzyme in mononuclear phagocytes stimulated with LPS in the absence or presence of CAP11, using a murine macrophage cell line RAW264.7.

2. Materials and methods

2.1. Materials

Anandamide was purchased from Cayman Chemicals (Ann Arbor, MI, USA); *N*-arachidonyl phosphatidylethanolamine was from Biomol International, L.P. (Plymouth Meeting, PA, USA); [ethanolamine 1-^{3}H] anandamide and [arachidonyl-5,6,8,9,11,12,14,15-^{3}H] *N*-arachidonyl phosphatidylethanolamine were from American Radiolabeled Chemicals, Inc. (St. Louis, MO, USA): 4-(*N*,*N*-dimethylaminosulfonyl)-7-(*N*-chloroformylmethyl-*N*-methylamino)-2,1, 3-benzoxadiazole (DBD-COCL) was from Tokyo Chemical Industry (Tokyo, Japan): lipopolysaccharide (LPS, *E. coli* O111:B4) was from Sigma Chemical Co., (St. Louis); Alexa Fluor-488 conjugated LPS was from Molecular

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Abbreviations: LPS, lipopolysaccharide; CAP11, cationic antibacterial peptide of 11-kDa; NAPE-PLD, *N*-arachidonyl phosphatidylethanolamine hydrolyzing phospholipase D; FAAH, fatty acid amide hydrolase; FCS, fetal calf serum; DBD-COCL, 4-(*N*,*N*-dimethylaminosulfonyl)-7-(*N*-chloroformylmethyl-*N*-methylamino)-2,1,3-benzoxadiazole; RT-PCR, reverse transcription-polymerase chain reaction

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Probes (Eugene, OR, USA). A 43-mer peptide of CAP11, G^1-I^{43} (G^1LRKKFRKTRKRIQKLGRKIGKTGRKVWKAWREYGQI-PYPCRI^{43}) were synthesized by a solid phase method on a peptide synthesizer (model PSSM-8; Shimadzu, Kyoto, Japan) by fluorenylmethoxycarbonyl chemistry, and purified, as described previously [11,14–16]. A neutralizing anti-CD14 monoclonal antibody (4C1) was kindly provided by Dr. Adachi (Tokyo University of Pharmacy and Life Science, Japan) [17].

2.2. Extraction and derivatization of anandamide

RAW264.7 cells were maintained in RPMI1640 (Sigma) containing 10% fetal calf serum (FCS; <0.03 ng/ml endotoxin, Equitech-Bio, Kerrville, TX, USA). RAW264.7 (80–90% confluent in 150 mm dish) were washed with serum-free medium, and incubated without or with LPS (10 ng/ml) in the absence or presence of CAP11 (100 and 1000 ng/ml) in RPMI1640 containing 2% FCS at 37 °C for 2 h. The cells and media were recovered, and immediately added with phenymethylsulfonyl fluoride (an inhibitor for anandamide-degrading enzyme, fatty acid amide hydrolase) at a final concentration of 100 μM. Extraction of anandamide was carried out with chloroform and methanol, according to Bligh and Dyer's method [18]. After centrifugation at $1000 \times g$ for 10 min, the organic phase containing total lipid was collected and dried by nitrogen gas stream. The residue was reconstituted in 100 μl acetonitrile, and subjected to reverse-phase HPLC using a JASCO System equipped with an TSK gel ODS 80Ts (75 × 4.6 mm; Tosoh, Tokyo, Japan) column and a UV detector (JASCO, Tokyo, Japan). Anandamide was separated with an isocratic elution solvent consisted of acetonitrile and water (8:2, v/v). The fractions corresponding to the elution time of authentic anandamide were collected, dried with nitrogen stream, and reconstituted in 50 μl acetonitrile. The anandamide-containing solution was then added with 50 μl of 10 mM DBD-COCL, and vortexed thoroughly, followed by heating at 60 °C for 2 h for derivatization. Derivatization was stopped by adding 100 μl water, and then the mixture was subjected to solid phase extraction using a Empore C-18 disk cartridge column (Sumitomo 3 M, Tokyo, Japan) preconditioned by 200 μl acetonitrile and 400 μl water. Then, the column was washed twice with 150 μl acetonitrile/water (40/60, v/v), and the derivatized anandamide was eluted with 200 μl acetonitrile/water (90/10, v/v). The aliquot (40 μl) was subjected to HPLC analysis for the quantification of anandamide [19].

2.3. Quantification of anandamide by HPLC

The HPLC system consisted of an L-2180 pump, an L-2480 fluorescence detector, a D-2500 integrator (Hitachi, Tokyo, Japan), and an analytical column TSK-gel ODS 80Ts (75 × 4.6 mm) (Tosho, Tokyo, Japan). Separation was performed with a gradient using the two eluents A (acetonitrile/water, 70/30, v/v) and B (acetonitrile); 0–5 min, isocratic 100% A; 5–35 min, a liner gradient from 100% to 33% of A and 0% to 67% B; 35–40 min, a liner gradient from 33% to 0% A and 67% to 100% B; 40–50 min, isocratic at 100% B; 50–60 min, a liner gradient from 0% to 100% A and 100% to 0% B.

2.4. Subcellular fractionation

RAW264.7 cells (80–90% confluent in 150 mm dish) were incubated without or with LPS (10 ng/ml) in the absence or presence of CAP11 (100 and 1000 ng/ml) in RPMI1640 containing 10% FCS at 37 °C for 2 h. To prepare membrane fraction containing anandamide-synthesizing and -degrading enzymes, subcellular fractionation was performed [20,21]. In brief, after the incubation, cells were washed, suspended in 1 ml of 50 mM Tris–HCl (pH 7.3) containing 0.25 M sucrose and 0.05% EDTA, and sonicated on ice (50 W, 10 s). Cell debris were pelleted by centrifugation at $1000 \times g$ for 5 min at 4 °C, and the supernatants were further centrifuged at $105000 \times g$ for 1 h. Resulting pellets (membrane fractions containing enzyme samples) were resuspended in 60 mM bis-Tris–propane (pH 8.0) containing 2 mM dithiothreitol, 0.1% Triton X-100 and 10 mM $CaCl_2$ for the assay of anandamide-synthesizing enzyme, and 50 mM Tris–HCl (pH 9.0) containing 1 mM EDTA for anandamide-degrading enzyme, respectively. The protein contents were determined with the DC Protein Assay regents (Bio-Rad, Hercules, CA, USA).

2.5. Assay for the anandamide-synthesizing enzyme activity

The anandamide-synthesizing enzyme activity was measured as described previously [20]. Membrane fractions (100 μg protein) were mixed with a substrate, [^{3}H] *N*-arachidonyl phosphatidylethanolamine (NAPE; 100 μM, 12000 dpm/nmol) in a total volume of 100 μl 60 mM bis-Tris–propane (pH 8.0) containing 2 mM dithiothreitol, 0.1% Triton X-100 and 10 mM $CaCl_2$, and incubated at 37 °C for 30 min. The reaction was stopped by the addition of 250 μl ice-cold methanol, and unreacted NAPE was precipitated for 15 min after the addition of 50 μl 0.2 M $ZrCl_2$–0.1 M NaOH and 75 μl 0.1 M NaH_2PO_4. Thereafter, the mixtures were centrifuged at $5000 \times g$ for 15 min, and aliquots of the supernatants (200 μl) were subjected to liquid scintillation counting.

2.6. Assay for the mRNA expression of N-arachidonyl phosphatidylethanolamine hydrolyzing phospholipase D (NAPE-PLD)

RAW264.7 cells (80–90% confluent in 150 mm dish) were incubated without or with LPS (10 ng/ml) in the absence or presence of CAP11 (100 and 1000 ng/ml) in RPMI1640 containing 10% FCS at 37 °C for 2 h. Thereafter, total RNA was extracted, and the expression of NAPE-PLD and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was analyzed by reverse transcription-polymerase chain reaction (RT-PCR). In brief, total RNA was purified using an RNeasy mini kit (Qiagen, Valencia, CA), and RT-PCR was performed using a ReverTra Dash RT-PCR kit (Toyobo, Osaka, Japan); cDNA was synthesized by reverse transcription of total RNA (1 μg) using ReverTra Ace reverse transcriptase and oligo(dT) primer. PCR amplification was performed with KOD dash *Taq* polymerase in a Thermal Cycler (Eppendorf AG, Hamburg, Germany) for 35 cycles of 10 s at 98 °C, 10 s at 53 °C, and 30 s at 74 °C (for NAPE-PLD) or 22 cycles of 10 s at 98 °C, 10 s at 60 °C, and 30 s at 74 °C (for GAPDH), using the following PCR primers: NAPE-PLD, 5′-CCAGCCATCAGC-CATGAGGGT-3′ and 5′-GGAGCCATCAGCCATGAGGGT-3′ [22], and GAPDH, 5′-ACCACAGTCCATGCCATCAC-3′ and 5′-TCCACCACCCTGTTGCTGTA-3′ (Toyobo). PCR products were resolved by 2% agarose gel electrophoresis, visualized by ethidium bromide staining, and quantified using LAS-3000 image analyzer and MultiGauge software (FUJIFILM Corporation, Tokyo, Japan).

2.7. Assay for the anandamide-degrading enzyme activity

The activity of an anandamide-degrading enzyme, fatty acid amide hydrolase (FAAH) was measured as described previously [21]. Membrane fractions (20 μg) were mixed with a substrate, [^{3}H]-anandamide (5 μM, 30000 dpm/nmol) in 50 mM Tris–HCl (pH 9.0) containing 1 mM EDTA in a final volume 200 μl, and incubated at 37 °C for 30 min. The reaction was terminated by the addition of 400 μl chloroform/methanol (1:1, v/v). Then, the mixtures were vortexed for 30 s, and centrifuged at $5000 \times g$ for 5 min. The upper aqueous layer (200 μl) containing the degraded product, [^{3}H]-ethanolamine, was collected and subjected to liquid scintillation counting.

2.8. Assay for LPS-binding

RAW264.7 cells (10^6/ml) were incubated with Alexa488-conjugated LPS (10 ng/ml) at 37 °C for 15 min in the absence or presence of CAP11 (100 and 1000 ng/ml) or an neutralizing anti-CD14 monoclonal antibody (4C1, 4 μg/ml) in RPMI1640 containing 10% FCS. Cells were then washed twice with ice cold PBS, and the LPS-binding was analyzed by flow cytometry [11].

2.9. Statistical analysis

Data are shown as the means ± S.D. Statistical significance was determined by one-way ANOVA with multiple comparison test (Prism, GraphPad Software, Inc., San Diego, USA), and the results were considered significant at $P < 0.05$.

3. Results

3.1. Production of anandamide

A murine macrophage cell line RAW264.7 is known to produce anandamide in response to LPS-stimulation [23]. To investigate the effect of cathelicidin peptide (CAP11) on anandamide production, we first examined the anandamide production from LPS-stimulated RAW264.7. As shown in

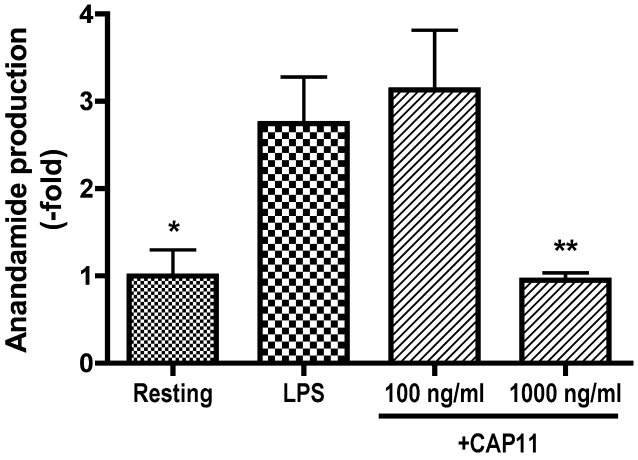

Fig. 1. Effect of CAP11 on the anandamide production from RAW264.7 cells. RAW264.7 cells were incubated without (Resting) or with 10 ng/ml LPS (LPS) in the absence or presence of CAP11 (+CAP11) at 37 °C for 2 h, and anandamide was measured by DBD-COCL-labeling and HPLC. Anandamide production is expressed as fold increase relative to Resting. Data are the means ± S.D. of three independent experiments. Values are compared between LPS and Resting or +CAP11. *, $P < 0.05$; **, $P < 0.01$.

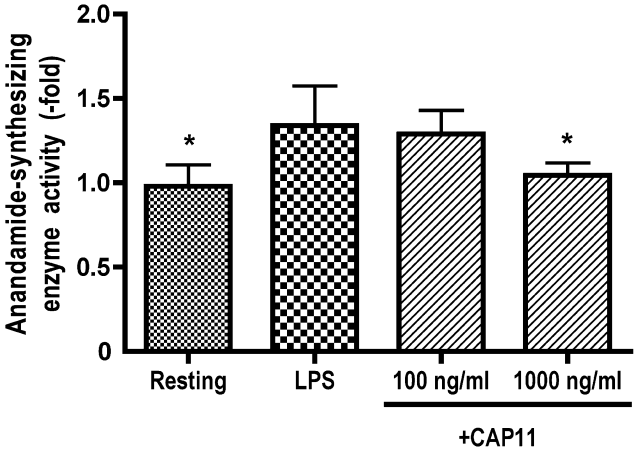

Fig. 2. Effect of CAP11 on the anandamide-synthesizing enzyme activity of RAW264.7 cells. RAW264.7 cells were incubated without (Resting) or with 10 ng/ml LPS (LPS) in the absence or presence of CAP11 (+CAP11) at 37 °C for 2 h. The anandamide-synthesizing enzyme activity was measured using membrane fractions and expressed as fold increase relative to Resting. Data are the means ± S.D. of five independent experiments. Values are compared between LPS and Resting or +CAP11. *, $P < 0.05$.

Fig. 1, LPS-stimulation (10 ng/ml) enhanced the anandamide production 2.7-fold; anandamide production was significantly increased from 3.06 ± 0.91 (Resting) to 8.41 ± 1.64 (LPS) pmol/10^8 cells ($P < 0.01$). Of note, 1000 ng/ml CAP11 markedly suppressed the anandamide production to almost the resting level ($P < 0.001$), although 100 ng/ml CAP11 hardly affected the LPS-induced anandamide production.

3.2. Anandamide-synthesizing enzyme activity

To clarify the suppressive effect of CAP11 on anandamide production, we next investigated the activity of anandamide-synthesizing enzyme. The activity was measured based on the release of [^{3}H]-anandamide from [^{3}H]-*N*-arachidonyl phosphatidylethanolamine, using rapid zirconium precipitation method [20]. As shown in Fig. 2, RAW264.7 cells has a basal anandamide-synthesizing enzyme activity, and LPS slightly but significantly enhanced the activity (1.3-fold, $P < 0.05$); the anandamide-synthesizing enzyme activity was increased from 0.26 ± 0.09 (Resting) to 0.33 ± 0.07 (LPS) nmol/min/mg protein. In accord with the effect on anandamide production, 1000 ng/ml but not 100 ng/ml CAP11 significantly suppressed the enzyme activity ($P < 0.05$). Thus, the inhibitory effect of CAP11 on anandamide production possibly depends on the suppression of anandamide-synthesizing enzyme activity.

NAPE-PLD was first isolated as an N-acylethanolamine phospholipid-degrading enzyme [24] and later known as an anandamide-synthesizing enzyme [22]. However, Liu et al. have recently reported that LPS-stimulated RAW264.7 produce anandamide through an alternative pathway independent of NAPE-PLD [25]. To confirm this issue, we examined the expression of NAPE-PLD mRNA after LPS-stimulation of RAW264.7 cells and also the effect of CAP11 on the mRNA expression. Consistent with the previous observation [25], the expression of NAPE-PLD mRNA was significantly downregulated (about 30%) by LPS-stimulation (Fig. 3), which increased the anandamide production from RAW264.7 cells (Figs. 1 and 2). Furthermore, the LPS-induced downregulation of NAPE-

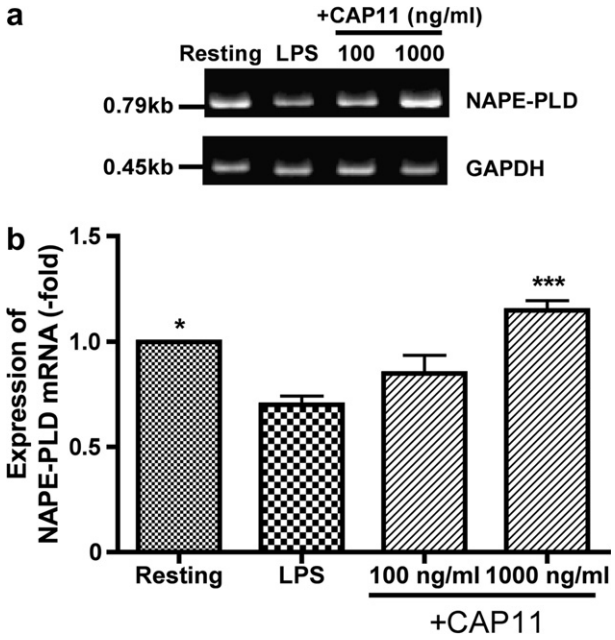

Fig. 3. Assay for the mRNA expression of *N*-arachidonyl phosphatidylethanolamine hydrolyzing phospholipase D (NAPE-PLD). RAW264.7 cells were incubated without (Resting) or with 10 ng/ml LPS (LPS) in the absence or presence of CAP11 (+CAP11) at 37 °C for 2 h. The expression of NAPE-PLD was detected using RT-PCR (a) and expressed as fold change relative to Resting (b). Data are the means ± S.D. of three independent experiments. Values are compared between LPS and Resting or +CAP11. *, $P < 0.05$; ***, $P < 0.001$.

PLD mRNA expression was reversed by 1000 ng/ml but not 100 ng/ml CAP11 (Fig. 3). These observations indicate that not NAPE-PLD but an alternative anandamide-synthesizing pathway plays a role in the increased production of anandamide (conversion of NAPE to anandamide) from LPS-stimulated RAW264.7 cells, as previously suggested [25], and that the suppressive effect of CAP11 on the LPS-induced ananda-

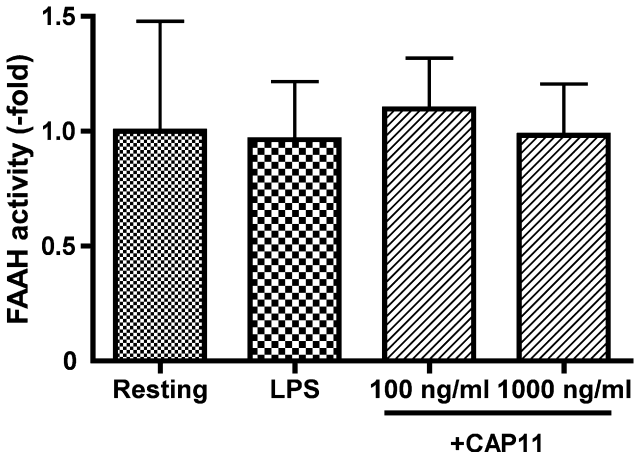

Fig. 4. Effect of CAP11 on the FAAH activity of RAW264.7 cells. RAW264.7 cells were incubated without (Resting) or with 10 ng/ml LPS (LPS) in the absence or presence of CAP11 (+CAP11) at 37 °C for 2 h. FAAH activity was measured using membrane fractions and expressed as fold change relative to Resting. Data are the means ± S.D. of four independent experiments.

mide production (anandamide-synthesizing enzyme activity) cannot be explained simply by the effect on NAPE-PLD.

3.3. FAAH activity

It is possible that the CAP11-mediated suppression of anandamide production is due to the enhanced activity of FAAH, an anandamide-degrading enzyme. Therefore, we investigated the effect of CAP11 on the FAAH activity. FAAH activity was measured based on the [^{3}H]-ethanolamine from [^{3}H]-anandamide. As shown in Fig. 4, in contrast to the effects on the synthetic enzyme, the FAAH activity was not affected by not only LPS but also CAP11 (0.96–1.1-fold change of the resting level); the FAAH activity was 0.12 ± 0.06 nmol/min/mg protein in resting cells, and was almost the same in LPS-stimulated or CAP11-treated cells. Thus, the CAP11-mediated suppression of anandamide production is unlikely to be due to the change in the activity of an anandamide-degrading enzyme FAAH.

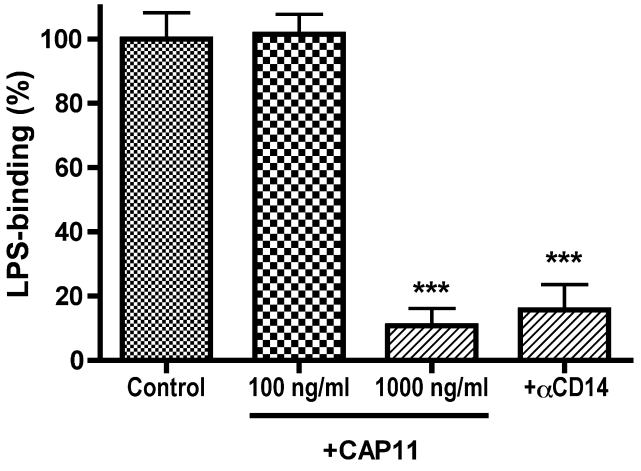

Fig. 5. Effect of CAP11 on the binding of LPS to RAW264.7 cells. RAW264.7 cells were incubated with 10 ng/ml Alexa488-conjugated LPS in the absence (Control) or presence of CAP11 (+CAP11) or an neutralizing anti-CD14 antibody (+αCD14) at 37 °C for 15 min, and then the LPS-binding was analyzed by flow cytometry. The LPS-binding is expressed as a percentage of Control (mean fluorescence intensity of 0.47 ± 0.06). Data are the means ± S.D. of three independent experiments. Values are compared between Control and +CAP11 or +αCD14. ***, $P < 0.001$.

3.4. LPS-binding to RAW264.7 cells

CAP11 has a potent LPS-neutralizing activity and protects mice from endotoxin shock [11]. To clarify the suppressive effect of CAP11 on anandamide production, we finally examined the effect of CAP11 on LPS-binding to RAW264.7 cells using Alexa488-conjugated LPS by flow cytometry. As shown in Fig. 5, in concordance with the effects on the anandamide production and NAPE-PLD activity, 1000 ng/ml but not 100 ng/ml CAP11 inhibited the LPS-binding. In addition, we confirmed that the LPS-binding was almost completely inhibited by a neutralizing anti-CD14 monoclonal antibody (4 μg/ml) [17], suggesting that CAP11 inhibits the CD14-mediated LPS-binding to RAW264.7 cells.

4. Discussion

Endotoxin shock is severe and abnormal conditions that are induced during serious infections with Gram-negative bacteria [1]. Endotoxin shock could also occur in the process of antibiotic therapy for the underlying bacterial infections in septic syndrome. In this context, some antibiotics are known to kill the bacteria but stimulate the release of LPS from outer membrane of dying bacteria, thereby provoking endotoxin shock [26]. Thus, a novel drug that suppresses the actions of LPS could be utilized for a reasonable adjunctive therapy against endotoxin shock or sepsis caused by Gram-negative bacteria.

CAP11, a cathelicidin peptide isolated from guinea pig has a strong antibacterial against Gram-positive and -negative bacteria [14,16]. CAP11 also exhibits a potent LPS-neutralizing activity, and rescues experimental animals from endotoxin shock [11]. Mononuclear phagocytes have been reported to produce anandamide in vitro upon stimulation with LPS [23]. Thus, in this study, to elucidate the protective mechanism of CAP11 for endotoxin shock, we evaluated the action of CAP11 on the production of anandamide, a key mediator of endotoxin shock in vitro using RAW264.7, a murine macrophage cell line.

LPS-stimulation induced the production of anandamide from RAW264.7 cells, accompanied with the enhanced anandamide-synthesizing enzyme activity, which is likely to depend on an alternative anandamide-synthesizing pathway rather than NAPE-PLD [25]; however, the activity of an anandamide-degrading enzyme (FAAH) was not affected by LPS-stimulation. Of importance, 1000 ng/ml CAP11 almost completely suppressed the LPS-induced anandamide production and the increase of anandamide-synthesizing enzyme activity, whereas 100 ng/ml CAP11 affected neither the anandamide production nor anandamide-synthesizing enzyme activity. Consistent with these actions, 1000 ng/ml but not 100 ng/ml CAP11 abrogated the LPS-binding to CD14 on RAW264.7 cells. These observations apparently indicate that CAP11 inhibits the binding of LPS to CD14-positive mononuclear phagocytes, thereby suppressing the increase of an anandamide synthesizing enzyme activity and the production of anandamide from the cells.

In the case of endotoxin shock patients, serum levels of anandamide are increased for three or four times of those in healthy volunteers [27]. Furthermore, anandamide has been proposed to exert cardiovascular actions as a contributor to endotoxin shock-induced hypotension [2], which results in circulating failure and multiple organ impairment in Gram-negative bacterial sepsis. The present study has demonstrated that CAP11 has a potential to suppress anandamide production based on its LPS-neutralizing activity. We previously revealed that CAP11 has a potent antibacterial activities against Gram-positive and -negative bacteria [14,16] and inhibits the production of cytokines from mononuclear phagocytes, which mediate the development of endotoxin shock [11]. Thus, CAP11 could be a promising candidate for adjunctive therapy in Gram-negative bacterial sepsis, since CAP11 could be expected not only to overcome the underlying bacterial infections but also to block the LPS-triggered inflammatory reactions by suppressing the binding of LPS to CD14-positive cells.

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